AF

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 1/113, 14/65, 14/61

A1

(11) International Publication Number: WO 97/18233

(43) International Publication Date: 22 May 1997 (22.05.97)

(21) International Application Number: PCT/SE96/01456

(22) International Filing Date: 12 November 1996 (12.11.96)

(30) Priority Data:
9504019-2 13 November 1995 (13.11.95) SE

(71) Applicant *(for all designated States except US)*: PHARMACIA & UPJOHN AB [SE/SE]; S-112 87 Stockholm (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GUSTAFSSON, Jan-Gunnar [SE/SE]; Rådjursstigen 6, S-756 53 Uppsala (SE). ÖHMAN, Johan [SE/SE]; Lövstavägen 48, S-194 42 Upplands Väsby (SE).

(74) Agents: TANNERFELDT, Agneta et al.; Pharmacia & Upjohn AB, S-112 87 Stockholm (SE).

(81) Designated States: AU, CA, JP, NO, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD FOR PRODUCING A CORRECTLY FOLDED, BIOLOGICAL ACTIVE RECOMBINANT PROTEIN

(57) Abstract

The present invention relates to a method for producing a correctly folded, biological active recombinant protein or polypeptide, comprising the steps of expression of the protein in prokaryotic cells, harvest of the cells, direct solubilization of the cells in a buffer at pH about 8 to 11 and thereafter dilution with water and a diluent. The protein or polypeptide is preferably GH, IGF-I or IGF-II.

METHOD FOR PRODUCING A CORRECTLY FOLDED, BIOLOGICAL ACTIVE RECOMBINANT PROTEIN

The present invention relates to a method for producing a correctly folded, biological active recombinant protein or polypeptide, comprising the steps of expression of the protein in prokaryotic cells, harvest of the cells, directly solubilization of the cells in a buffer at pH about 8 to 11 and thereafter dilution with water and a diluent.

The protein or polypeptide is preferably GH, IGF-I or IGF-II.

INTRODUCTION

A general, major problem when recombinant proteins are overproduced in efficient bacterial expression systems is related to the folding of the protein products into their native conformations. Many high level expression system in *Escherichia coli* results in the production of aggregates of denatured proteins, so called inclusion bodies, which in some cases may be refolded into the wanted native protein. General methods to facilitate and render the refolding effective has been found. One is the use of a class of heat-shock-proteins (HSP) and the other is folding-enzymes. By using HSP, aggregation is avoided and by using the folding enzymes, the speed of refolding is accelerated.

However, not all protein are susceptible for these methods and other solutions to enhance refolding yields have been suggested. In an article by J D Carlson et al in Biotechnology, Vol 10, January 1992, the use of monoclonal antibodies during protein refolding, to enhance the yield of native protein, especially S-Protein, has been disclosed.

Another suggested method for the recovering of the native protein is solubilization of the inclusion body protein with a denaturant, such as guanidine or urea and if needed a reduction of the disulphide bond. By dilution or dialysis and reoxidation, the protein can be refolded to the native protein.

Successful refolding, without formation of new inclusion bodies, is generally difficult at high concentrations of the recombinant protein. The best yield is generally achieved at concentrations around 20-200 µg/ml. Refolding is therefore considered to be a very expensive production form that demands a cost intensive drug.

However, the yield of a refolding procedure is unpredictable since the protein product often aggregates or gets modified. In addition, for IGF-I and II, the soluble refolded fraction will contain misfolded species and the overall yield of correctly folded growth factor is rather low (Samuelsson, E., et al (1991) Bio/Technology Vol. 9, Page 363).

In order to increase the yield of correctly folded IGF -I different methods have been proposed.

Human insulin-like growth factor I (IGF-I) is a single-chain peptide growth factor of 70 amino acids, originally isolated from serum. IGF-I is positively regulated by growth hormone (GH) and shows mitogenic effects on many cell types. Therefore, IGF-I is thought to mediate many of the growth promoting effects of GH. In the regions of homology, IGF-I and insulin are 49% homologous, including the six cysteine residues, furnishing three disulphide bridges . The three dimensional structure of IGF-I has been modelled based on the x-ray structure of insulin, and this model has recently been confirmed in the disulphide bridge regions by distance constraints obtained by 2-D NMR spectroscopy of IGF-I (for a review on

IGF, see: Insulin-like growth factors I and II, Humbel R. E, Eur. J. Biochem 190, 445-462,1990).

Human recombinant IGF-I has been produced as a secreted product in both *Escherichia coli* and *Saccharomyces cerevisiae*. In isolated material from both species, IGF-I is found mainly as miss-folded forms with intermolecular disulphides. In addition, *in vitro* refolding of reduced IGF-I in the presence of oxygen, has demonstrated that native, miss-matched and aggregated IGF-I accumulate, even under dilute refolding conditions.

The refolding yield of recombinant IGF-I was significantly improved by utilising a fused fusion partner, consisting of two IgG-binding domains (ZZ) derived from staphylococcal protein A (Samuelsson, E., et al (1991) Bio/Technology Vol. 9, Page 363). The ZZ fusion partner is used to solubilise misfolded molecules before, during and after reduction and reoxidation. The yield of correctly folded IGF-I is shown to be substantially increased but there is still a significant amount of misfolded IGF.

Patents and patent applications have also described the problem of misfolded IGF and suggested different improvements.
WO 91/02807 (Amgen) (=US 5158875) discloses a method for refolding IGF-I in the presence of a fused short positively charged leader sequence, in which amino acids, such as lysine, arginine and histidine are fused at the N-terminus of IGF-I. Inclusion bodies are isolated and solubilized with urea.
In WO 93/11240 (Genentech) a method for refolding of insoluble and improperly folded IGF-I is described involving solubilisation of inclusion bodies and refolding in a single buffer system.
US 5 151 501 (American Cyanamid) discloses a process for solubilization and naturation of somatropins (Growth hormones) by dispersing

somatropin refractile bodies in a solution containing sulfolane and thereafter dilution.

WO 9319084 (Synergen) discloses a method for producing active IGF-I is claimed, comprising the steps of expressing in prokaryotic cell, adding a first reducing agent, adding denaturing agent (e.g. urea), adding oxidizing agent (e.g. oxidized gluthatione or cystein) and adding a second reducing agent (e.g. DTT, cystein etc.). Met-IGF-I is expressed.

WO 9506064 discloses a process for increasing the yield of correct refolding of a polypeptide and in which a copper or manganese salt are present during the refolding step and WO 9506059 (Genentech) discloses a method for isolation of cells by adding a phase-forming species to form mutilple aqueous phase.

We have now invented a novel, simplified method for the production of correctly folded biological active recombinant protein or polypeptide. Especially we refer to recombinant IGF-I after expression of Z- IGF-I. Reference is here given to EP 230 869, especially the examples.

With *Escherichia coli* expressing the hybrid protein Z-IGF-I, we have achieved very high expression levels (up to 15 g/l fermentation).

Although the method here is described with IGF-I as the preferred polypeptide, it can also be used for recombinant preparation of other polypeptides, when misfolded species and the overall yield of correctly folded polypeptide is rather low.

With our claimed method we can avoid the steps of mechanical disruption of the cells and the isolation and washing of refractile bodies.

Our process is easier than the earlier described and gives a good yield.

THE INVENTION

The invention relates to a method for producing a correctly folded, biological active recombinant protein or polypeptide, comprising the steps of

a) expression of the protein in prokaryotic cells,

b) harvest of the cells

c) directly solubilization of the cells in a buffer at pH about 8 to 11, , preferably about 8, with a chaotropic agent and a reducing agent and

d) dilution with water and a diluent

The protein could e.g. be IGF-I, IGF-II or GH.

When the protein is IGF-I the method preferably comprises the steps of

a) expression of an IGF-I-fusion protein in prokaryotic cell system, preferably E Coli,

b) harvest of the cells

c) directly solubilization of the cells in a buffer at pH 8 to 11, preferably about 8, with a chaotropic agent and a reducing agent

d) dilution with water and a diluent

e) addition of a cleaving agent and

f) purification to produce the biological active IGF-I.

The IGF-I-fusion protein is preferably a hybrid Z-IGF-I. The buffer in step c) could be e.g. Tris (Tris [hydroxymethyl]aminomethane hydrochloride) or glycin.

The chaotropic agent in step c) is preferably guanidine or urea and guanidine could be used in a concentration of 3-7 M, preferably 5M.

The reducing agent in step c) could be e.g. DTT (DL-Dithiothreito) or cystein and the diluent in step d) could be ethanol.

After step d), pH is preferably reduced below pH 6 and more preferably to pH 3 or below.

For the preparation of IGF-I pH is preferably reduced to pH 3 or below between steps d) and e).

A concentration step and a buffer exchange between steps d) and e), in which chromatography and/or ion-exchange preferably is used is also claimed.

The cleaving agent in step e) in the preparation of IGF-I could be hydroxylamine, an enzyme or any other cleaving agent.

The purification steps for the preparation of the pure protein or polypeptide include e.g. cation exchange, RP-HPLC and/or hydrophobic interaction Chromatography (HIC).

When IGF-I is produced as Z-IGF-I, IGF-I has a better solubility , which means that we can have a higher concentration of reduced IGF-I in solution, giving a high amount of right folded IGF-I which is of most importance for an industrial method for the production of IGF-I.

EXAMPLES

The recombinant human IGF-I (rhIGF-I) used in the experiments was produced in E Coli according to the method described in EP 230 869, example VIII (but in a fermentor) including growing at 37° C for 20 hours.

Example 1

Solubilization

The cell was harvested after fermentation by centrifugation or cross flow filtration.

Thereafter the cells were dissolved in:

13 L cell solution, wet weight 498 g/L
5 mol/L guanidine-hydrochloride
97 mmol/L Tris-base
159 mmol/L Tris-HC1
2 mmol/L ethylene-dinitro-tetraacetic acid-disodiumsalt-dihydrate (EDTA)
4 mmol/L dithiothreitol (DDT)
Total volume: 16.9 L

The pH was kept at 8.1, the solubilization was run for 3 hours under stirring at 15° C.

Refolding

The solubilization solution was diluted with 33.8 L 22.5% ethanol solution, dilution factor 3.

The pH was kept at 8.1, under stirring at 15°C.

The refolding was stopped after 20 hours by addition of concentrated hydrochloric acid until the pH of the solution was <3.1.

RP-HPLC analysis of the concentration of Z-IGF-I in the refolding solution gave correctly folded 3.249 g ZIGF-I/L.

Example 2

Solubilization

The cell was harvest after fermentation by centrifugation or cross flow filtration.

Thereafter the cells were dissolved in:

154 mL cell solution, wet weight 450 g/L
4.5 mol/L guanidine-hydrochloride
400 mmol/L glycin
0.2% Tween 20
2 mmol/L ethylene-dinitro-tetraacetic acid-disodiumsalt-dihydrate (EDTA)
3 mmol/L dithiothreitol (DDT)
Total volume: 200 mL

The pH was kept at 10.0, the solubilization was run for 3 hours under stirring at room temperature.

Refolding

The solubilization solution was diluted with 125 mL ethanol, 400 mL water, 66.8 g guanidine-hydrochloride, dilution factor 4.

The pH was kept at 10.0, under stirring at room temperature.

The refolding was stopped after 20 hours by addition of concentrated hydrochloric acid until the pH of the solution was <3.1.

RP-HPLC analysis of the concentration of Z-IGF-I in the refolding solution gave correctly folded 1.38 g ZIGF-I/L.

Example 3

Solubilization

The cell was harvested after fermentation by centrifugation or cross flow filtration.

Thereafter the cells were dissolved in:

900 mL cell solution, wet weight 720 g/L
6 mol/L guanidine-hydrochloride
97 mmol/L Tris-base
159 mmol/L Tris-HC1
2 mmol/L ethylene-dinitro-tetraacetic acid-disodiumsalt-dihydrate (EDTA)
3 mmol/L dithiothreitol (DDT)
Total volume: 1.17 L

The pH was kept at 8.0, the solubilization was run for 3 hours under stirring at 15°C.

Refolding

The solubilization solution was diluted with 547 mL ethanol 1786 mL water, dilution factor 3.

Total volume: 3.6 L

The pH was kept at 8.1, under stirring at 15°C.

The refolding was stopped after 21 hours by addition of concentrated hydrochloric acid until the pH of the solution was <3.1.

RP-HPLC analysis of the concentration gave correctly folded 1.32 g Z-IGF-I/L.

Yield

Solubilization: 74% (of ZIGF-I from the fermentor)

Total, solubilization and refolding: 41%

Finally purified IGF-I was shown to have biological activity in e.g. chick embryo femore and RRA (Radio Receptor Assay).

Example 4, first purification step

A solution from the refolding step was diluted with WFI (Water for Injection), dilution factor 3 and clarified on a cross flow membrane before applied on a cation exchanger (Pharmacia Biotech BPG 200/500, SP-Sepharose FF, 9.5 Liters, 0.33 m bed high).

The following buffers were used:

Step	Buffer	pH
Equilibration	100 mM Citric acid/di-Na hydrogen phosphate	3.0
Wash 1	50 mM Citric acid/di-Na hydrogen phosphate	2.9
Wash 2	100 mM di-Na hydrogen- and Na-dihydrogen phosphate	5.9
Elution	100 mM Na hydrogen phosphate	7.5
Regeneration	500m M NaOH	
Sanitation	100 mM NaOH	

The column was washed with 1 Column volume (CV) WFI, the column was treated with 3 CV of Equilibration buffer and the sample was hereafter applied on the column.

The column was first washed with 3 CV of Wash 1 buffer and thereafter with 4 CV Wash 2 buffer.
The product was eluted with 5 CV of elution buffer and the column treated with 2 CV of Regeneration buffer and 2 CV of Sanitation buffer.

According to RP-HPLC the yield over the purification step was 98 % correctly folded IGF-I.

Example 5, cleavage step

After the first purification step on a cation ion exchange column, the cleavage of Z from IGF-I was performed.
To a vessel was added : 40.0 Liters from the cation ion exchanger pool, 1428 grams Sodium diphosphate and 4.0 Liters Hydrozylamine, 50 % sol.
The pH was adjusted to 9.55 (with NaOH) and the temperature was kept at 40° C.
After 3 hours the reaction was stopped by lowering the temperature to 25° C and adding 40.0 Liters concentrated Acetic acid (HAc) and 160 Liters WFI.
The pH was adjusted to 3.30.

According to RP-HPLC the yield over this step was 84 % of correctly folded, non oxidized IGF-I.

Example 6, cleavage step

After the first purification step on a cation ion exchange column, the cleavage of Z from IGF-I was performed.
To a vessel was added : 18.6 Liters from the cation ion exchange pool, 664 grams Sodium diphosphate and 2.17 Liters Hydrozylamine, 50 % sol.
The pH was adjusted to 9.5 (with HAc) and the temperature was kept at 40° C.
After 3 hours the reaction was stopped by lowering the temperature to 25° C and adding 22.3 Liters concentrated HAc and 93 Liters WFI.
The pH was adjusted to 3.35.

According to RP-HPLC the yield over this step was 61 % of correctly folded, non oxidized IGF-I.

CLAIMS

1. A method for producing a correctly folded, biological active recombinant protein or polypeptide, comprising the steps of

a) expression of the protein or polypeptide in prokaryotic cells

b) harvest of the cells

c) directly solubilization of the cells in a buffer at pH about 8 to 11 with a chaotropic agent and a reducing agent and

d) dilution with water and a diluent.

2. A method according to claim 1 in which the protein is IGF-I, IGF-II or GH.

3. A method according to claim 1 in which the protein is IGF-I and which comprises the steps of

a) expression of an IGF-I-fusion protein in prokaryotic cell system, preferably E Coli

b) harvest of the cells

c) directly solubilization of the cells in a buffer at pH 8 to 11 with a chaotropic agent and a reducing agent

d) dilution with water and a diluent

e) addition of a cleaving agent and

f) purification to produce the biological active IGF-I.

4. Method according to any of claims 1 to 3 in which the IGF-I fusion protein is hybrid Z-IGF-I.

5. Method according to any of claims 1 to 4 in which the buffer in step c) is Tris or glycin.

6. Method according to any of claims 1 to 5 in which pH in step c) is about 8.

7. Method according to any of claims 1 to 6 in which the chaotropic agent in step c) is guanidine or urea.

8. Method according to claim 7 in which guanidine is used in a concentration of 3-7 M.

9. Method according to any of claims 1 to 8 in which the reducing agent in step c) is DTT or cystein.

10. Method according to any of claims 1 to 9 in which the diluent in step d) is ethanol.

11. Method according to any of claims 1 to 10 in which pH is reduced after step d), preferably below pH 6 more preferably to pH 3 or below.

21. Method according to claim 11 in which pH is reduced to pH 3 or below between steps d) and e).

13. Method according to claim 3 in which there is a concentration step and a buffer exchange between steps d) and e).

14. Method according to claim 13 in which chromatography and/or ion-exchange is used.

15. Method according to any of claims 1 to 14 in which cleaving agent in step e) is hydroxylamine.

16. Method according to claim 14 in which purification step f) include cation exchange, RP-HPLC and/or hydrophobic interaction Chromatography (HIC).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/01456

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 1/113, C07K 14/65, C07K 14/61
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REG, CAPLUS, WPI, DPCI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9506064 A1 (GENENTECH, INC.), 2 March 1995 (02.03.95) --	1-16
A	WO 9506059 A1 (GENENTECH, INC.), 2 March 1995 (02.03.95) --	1-16
A	Bio/Technology, Volume 12, November 1994, Roger A. Hart et al, "Large Scale, In Situ Isolation of Periplasmic IGF-I from E. coli" page 1113 -- --------	1-16

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" ertier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 March 1997	05-03-1997

Name and mailing address of the ISA/	Authorized officer
Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Carolina Gómez Lagerlöf Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/SE 96/01456

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9506064	02/03/95	CA-A- 2168552	02/03/95
		EP-A- 0714406	05/06/96
WO-A1- 9506059	02/03/95	AU-B- 673624	14/11/96
		AU-A- 7561694	21/03/95
		CA-A- 2167910	02/03/95
		EP-A- 0714403	05/06/96
		US-A- 5407810	18/04/95